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RESEARCH ARTICLE

Combined effect of Lead acetate and Zinc chloride on the Fitness of *Drosophila melanogaster*

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Abstract

Heavy metals have been considered as one of the key environmental toxicants with a wide range of health effects on humans. The bulk of information available today is mainly focused on the single toxin studies with only few studies enumerating the synergistic, additive or protective effects of these heavy metals. In current study, *Drosophila* was used as a model to investigate the effects of two prominent heavy metals- Lead (Pb) and Zinc (Zn). The developmental time, viability and fertility of these fruit flies after a combined and single exposure to lead acetate and/or zinc chloride were assessed with a focused aim of learning if the combined exposure at sub- lethal concentrations provokes synergistic changes in the fitness of *Drosophila melanogaster*. Larval feeding method was employed throughout the developmental period of the experimental subjects to administer the compounds singly or in combination while maintaining multiple replicates in order to minimize experimental error. Subsequently, number of offspring produced, their eclosion time and viability were recorded. Our results show that developmental exposure of sub lethal concentrations of lead acetate and zinc chloride provokes disturbances in the fitness of flies. A delay in developmental time was shown which is far more pronounced for the combined exposure than single lead intoxication. A synergistic effect was seen on the developmental viability with a survivability of only 10- 28% at combined exposure of zinc and lead. Further in contrast to the prior parameters, combined treatment showed a significant increase in fertility when compared to single exposure of lead acetate-suggesting a protective effect of Zinc over Lead with respect to fertility.

INTRODUCTION

Thousands of chemicals are in commercial use today, with more being introduced each year for which there is little or no toxicological data. Furthermore, we continue to be exposed to the increasing environmental stress caused by persistent toxicants without any knowledge of its harmful effects (Rand, 2010) Heavy metals form a major causative group with cognitive and neurological implications of exposure. Excess accumulation of few heavy metals like Copper and Zinc which are otherwise of nutritional and physiological importance; and non- essential toxic metals like Lead and Mercury is detrimental (Hapke *et al.* 1987; Yepiscoposyan *et al*. 2006). Studies have shown that the effects of chronic developmental exposure of these toxins are more pronounced than the acute adult exposure (Hirsch et al., 2003). This may be due to its direct effects on the developing neuronal and endocrine systems that regulate fitness and behaviour in adults. Even sub- lethal doses of toxins are capable of altering the physiological mechanisms qualitatively and quantitatively as shown by Hirsch et al (2003) in fruit flies.

Compared to several other metal ions with similar chemical properties, Zinc is less toxic under physiological concentrations but at higher concentrations may bind to inappropriate sites in proteins or cofactors and interfere with their functions; thus exhibiting zinc intoxication (Laura et al, 2010) In addition, consumption of larger doses of supplemental zinc for extended periods has been associated with copper deficiency (Magee and Matron, 1960; Ogiso *et al*, 1974), impaired immune function and neuronal death (Chen and Liao, 2003) Lead, unlike zinc, is toxic even at very low concentrations posing a significant health risk. In a recent review by Neal and Guilarte (2012) it has been cited that the biological fate of lead depends on various factors like age, nutrition, health etc. It can interact with Zinc ions which are essentially present in the body and induce cognitive and behavioral deficits in adults and children.

Today, though a bulk of information is available, most studies that enumerate the effects of various toxins focus on single toxin. It has been suggested by Singh et al (2010) that protective or synergistic effects can arise due to combination of toxins. Hence there is a considerable interest in understanding the combined effects of heavy metals. The fruit fly, *Drosophila* is a convenient system to address this question since many aspects of metal homeostasis are conserved between flies and humans (Bonneton et al, 1996). It is an ideal model to conduct preliminary studies like fitness analysis. Net fitness comprises various components such as viability, female fecundity, male mating ability, developmental time, longevity, etc (Yamazaki, 1984) Fitness plays a central role in evolutionary biology which has inspired numerous treatments of this concept from both theoretical and experimental points of view (Hamer and Hartl, 1983) Experimentation with *Drosophila* has been quite diverse, ranging from the measurement of a single component of fitness in individual flies to estimates of overall fitness based on the long-term reproductive success (Hamer and Hartl, 1983) Since fitness is majorly regulated by dietary and environmental factors, it is susceptible to changes associated. Stress acts as the key factor in reducing fitness of an organism and heavy metal intoxication is one of them (Neethu et al, 2014) *Drosophila* facilitates the easy manipulation and analysis of this parameter thereby providing us the insights that can be utilized in the process of understanding the complex homeostatic mechanisms of human body.

In our present study, we have considered two heavy metals- Lead acetate that has unavoidable environmental exposure and Zinc chloride which has inappropriate dietary exposure in present day life. The study was conducted to assess the single and combined effects of Lead acetate and Zinc chloride on the 3 vital components of fitness- developmental time, viability and fertility in *Drosophila melanogaster* by hypothesizing that excess dietary intake of Zinc chloride can add to the toxic effects of lead acetate.

2. MATERIALS AND METHODS:

Fly culture: Wild type *Drosophila melanogaster- Oregon K* strain (*OK*) was obtained from *Drosophila* stock centre, University of Mysore. Flies were grown and aged in culture bottles/vials on wheat cream agar media (100 Soji, 100g jaggery, 10g agar and 7.5ml propionic acid in 1dm^3 distilled water) with regular subculturing and maintained for all experiments at 24 °C with 60-70% relative humidity and ambient lighting condition with a sprinkle of live Baker's yeast. All collection of virgin, adult flies were performed under brief anaethesia. Dose administration was achieved via larval feeding for all treatments.

2.1: Determination of LC50: For the convinient establishment of treatment groups, LC_{50} was first determined. Eggs collected from the OK cultures by modified method of Delcour (Ramachandra and Ranganath, 1988) were distributed on the culture medias with 0.625mM, 1.25mM, 2.5mM, 5mM and 10mM Lead acetate (SDFCL, Mumbai) and 0.625mM, 1.25mM, 2.5mM, 5mM and 10mM, 20mM and 40mM Zinc chloride (RFCL, New Delhi). 5 replicates per concentration were maintained for both compounds. The eggs that developed into lavae were noted and unhatched eggs were omitted from the experiment. Total flies emerging were recorded and mortality was calculated in percentage as (no. of eggs hatched- no. of adults emerged) x 100. LC_{50} was determined from probit analysis and two sub lethal concentrations were considered for experimental purpose at which approximately 20% (T1) and 30% (T2) mortality was observed for both compounds. A third set of treatment group (combined group) was introduced with T1 concentrations of Lead acetate- zinc chloride and T2 concentrations of Lead acetate- zinc chloride (Table 1)

2.2: Fitness: To determine the developmental time and viability, eggs were collected from experimental flies as mentioned before, at 4hrs interval and equally distributed into different treatment media (150 each); control was maintained on zinc and lead free media. The total number of eggs that developed into adults and the time taken for this development was noted for 15 replicates per treatment group.

To assess the effect on fertility of flies, a pair of lead acetate treated flies was crossed in fresh food vial and maintained for 10 days. The total number of progeny produced in 10 days was recorded. The same was conducted for Zinc treatment, combined treatment and control with 10 replicates per group.

2.3: Statistics: Both logit and probit analysis was conducted to fit the probit dose response curves and calculate CI for dose response quantile LC_{50} and LC_{90} following a Chi- square (χ^2) distribution. Data for fitness parameters was analysed using SPSS 14.0 software with p<0.05 taken to indicate the significance statistically. The effect of Lead acetate and Zinc chloride singly and in combination, was assessed by One way Analysis of Variance (ANOVA) followed by Tuckey's HSD test for post- hoc comparisons. The correlation between the experimental groups (Lead, Zinc and Combined) was measured using Pearson's co-relation and significance was indicated at 0.01 level.

3. RESULTS

3.1. LC_{50} for lead acetate and Zinc chloride

The χ^2 values for both compounds obtained by probit analysis are significant at p<0.05 (Table 2). Having accepted the models are reasonable, it was concluded with 95% confidence that the true value for median concentration of Lead acetate lies between 2.78- 3.73mM and that of Zinc chloride lies between 9.13- 26.3mM at given conditions. Probit analysis yielded a LC_{50} of 3.21mM for lead acetate and of 14.23 for zinc chloride at given culture conditions. Logistic regression applied for the data produced sigmoid type curves with similar LC_{50} values (Figure 1). For experimetal purpose, the concentrations of both compounds which showed approximately 20% and 30% mortality were taken singly and in combination (shown inTable 1)

3.2. Fitness

3.2(a): Developmental time: For control groups, eclosion was seen from 10- 13 days post egg laying with a mean emergence on 11th day. Whereas for 1mM and 1.5mM lead acetate treatments the emergence was seen between 11- 14days showing a delay of 1 and 2 days in their mean developmental time for T1 and T2 respectively . Emergence was from 11 to 14th day for both treatments of zinc chloride with mean of 11th day as in control group. Significant delay was observed with flies developed on combination media which emerged between 13 to 17days with a mean egg to adult developmental time of 15th and 16th day post egg laying for T1 and T2 respectively (Table 3, Fig. 2(a))

3.2(b): Developmental viability: The effect of lead acetate, zinc chloride and their combination on survivability of *D. melanogaster* was measured based on the ability of eggs to emerge as adults. The percentage developmental viability of the *D. melanogaster* for sub lethal concentrations of Lead acetate and Zinc chloride is given in Table 3 and Fig 2(b). Controls showed survivability of 99.06% which decreased to 83.8% and 69.4% for Lead treatments. The decrease in the viability for Zinc treatments was small in magnitude between the treatments (T1= 81.4%, T2= 78.9%) but statistically significant with respect to control. Our results also indicated a greater decrease in the viability at increasing concentrations when lead and zinc were present together in the food media with survival rate of only 10- 28%. One- way ANOVA showed significant effect of concentration on viability for Lead (F= 4349.6, p<0.05), Zinc (F= 3122, p<0.05) and combinatorial treatments (F= 35531.8, p<0.05). According to Pearson's Co-relation, values obtained for Lead and Zinc treatment were significantly related to the values of combinatorial treatment group (Lead: r= 0.951; Zinc: r= 0.996, p<0.01) showing increased mortality with combination of both metal compounds than single treatment.

3.2(c): Fertility: Average offsprings produced by a treated female for 10 days after successful mating with a treated male are shown in Table 3. The Control (untreated pair) showed a mean fertility of 227.8± 2.2. Decrease in the fertility was noted for Lead treatment with an intensifying effect as the concentration increased (Fig 2(c)). 1mM caused a 16.82% decrease and 1.5mM showed 30.56% decrease in fertility. However, lower concentration of $ZnCl_2$ (5mM) showed statistically insignificant reduction and higher concentration of 7mM increased the fertility by 37%.

Like Lead, combined treatment showed reduction in fertility with increasing concentration but in a lesser magnitude than the Lead treatment (T1= 2.46%, T2= 11.11% decrease) One way ANOVA shows a significant difference in fertility among the experimental groups ($^{Lead}F_{2,27} = 417.5$, $^{Zinc}F_{2,27} = 712.1$, $^{Combination}F_{2,27} = 40.1$) at $p<0.05$. Tuckey's Post Hoc comparison showes that the difference in fertility between control, T1 and T2 is significant for Lead and combined treatment. No significant result was obtained for control and ZincT1 but contrary with ZincT2. The Pearson's Co-relation employed to relate the effect between Lead, Zinc and Combined groups showed a stronger positive linear relationship between Lead and combined treatment ($r= 0.886$, $p< 0.01$) and a negative co-relation for Zinc and combination ($r= -0.637$, $p<0.01$)

Table 1: Experimental groups with their respective compound concentrations

Experimental (E.) groups	Lead acetate	Zinc chloride	Combination
Treatment (T.) groups	Control	Control	Control
	T1- 1mM	T1- 5mM	T1- (1+5) mM
	T2- 1.5mM	T2- 7mM	T2- (1.5+7) mM

Table 2: Effect of Lead acetate and Zinc chloride on D. melanogaster (probit analysis)

Compound	LC50 (mM)	(95%FL)	LC90 (mM)	(95%FL)	Slope± S.E.	Heterogeneity X^2 (df)	Regression equation	Probability
Lead acetate	3.21	2.78-3.73	13.96	10.75- 19.78	2.01± 0.16	5.18 (3)	Y= 2.0101 X + 3.9801	0.158
Zinc chloride	14.23	9.13-26.3	80.86	38.91- 386.5	1.69± 0.21	9.1 (3)	Y= 1.6987 X+ 3.0408	0.028

Table 3: Mean developmental time, viability and fertility of normal and treated flies

		Developmental time (Days)	Developmental viability (No. of adults eclosed)	Fertility (No. of offsprings produced)
E. group	T. group	Mean± S.E.	Mean± S.E.	Mean± S.E.
Lead acetate	C	11.13±.0364	148.60± .244	227.80± 2.269
	T1	11.75±.0598	125.80± .374	189.50± 1.335
	T2	12.96±.0462	104.20± .374	158.20± 1.339
	ANOVA		*F2,12= 4349.6	*F2,27= 417.5
Zinc chloride	C	11.13±.0364	148.60± .244	227.80± 2.269
	T1	11.18±.0507	122.20± .374	222.30± 1.483
	T2	11.19±.0316	118.40± .244	312.20± 1.948
	ANOVA		*F2,12= 3122	*F2,27= 712.1
Combination	C	11.13±.0364	148.60± .244	227.80± 2.269
	T1	15.47±.1778	42.60± .400	233.10± 19.584
	T2	16.35±.1990	15.00± .447	202.50± 2262
	ANOVA		*F2,12= 35531.8	*F2,27= 40.1

*p<0.05

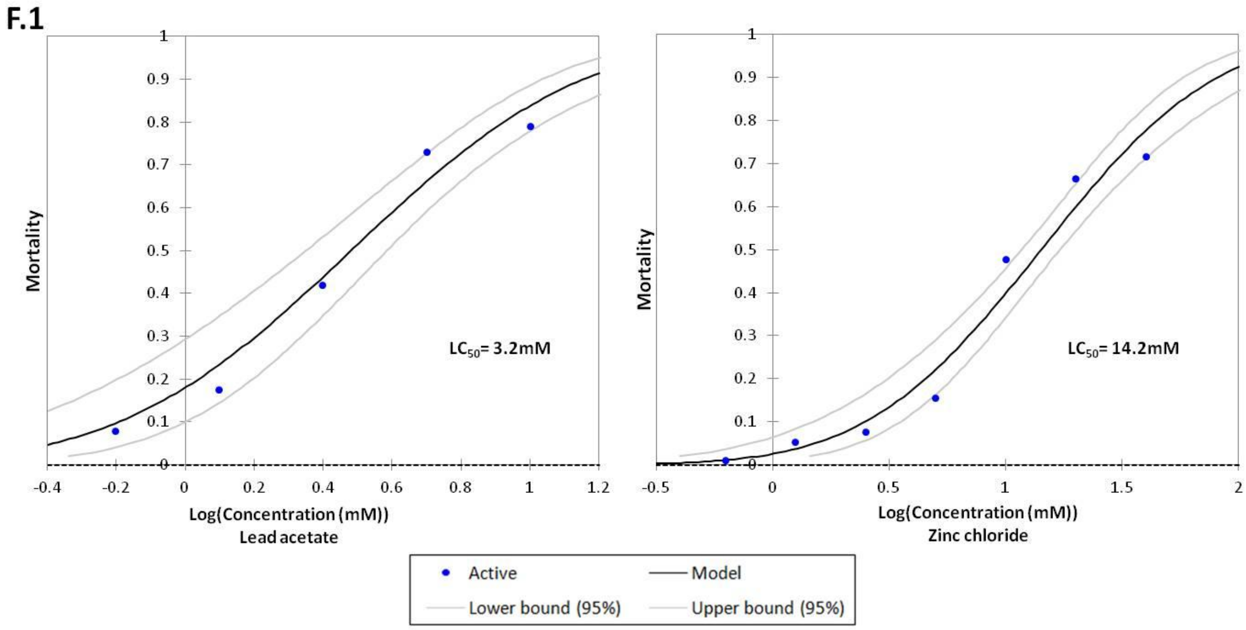
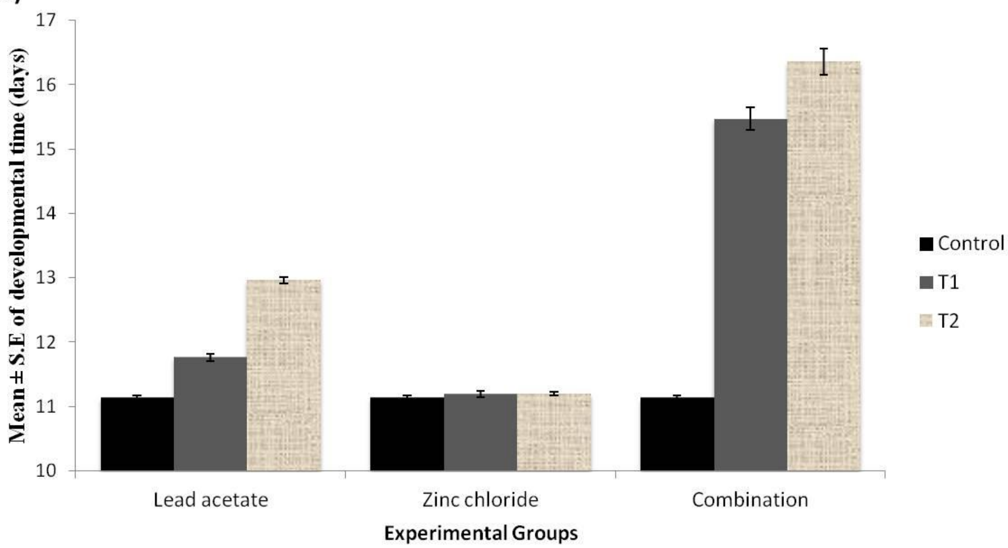
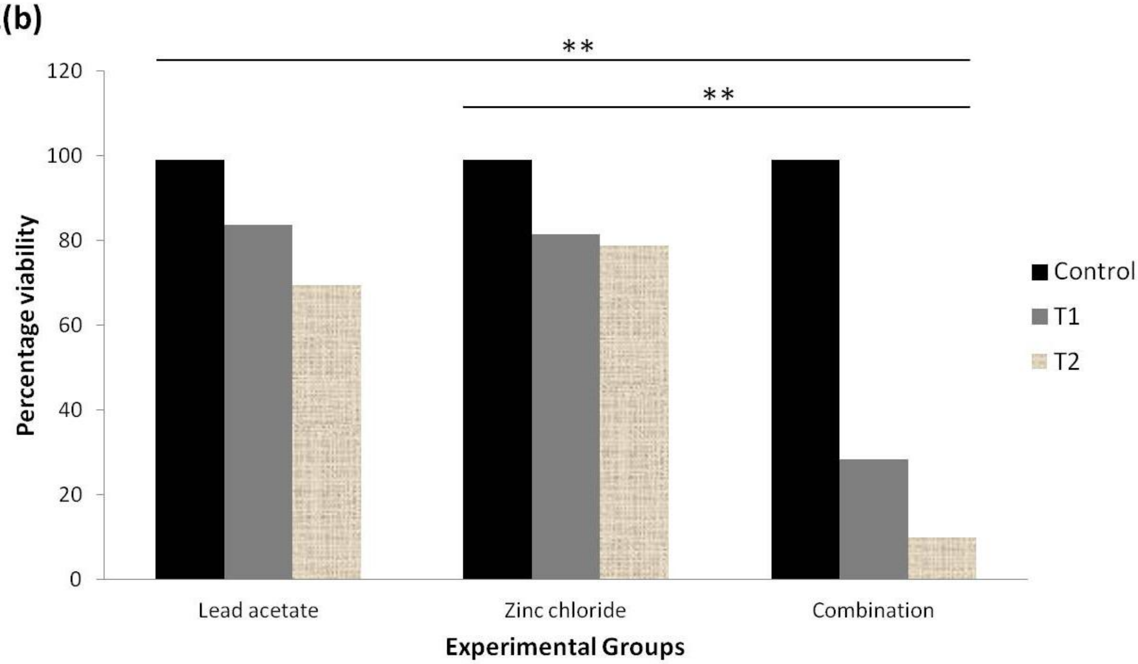


Fig. 1: Logistic regression of mortality of D. melanogaster by log (Concentration) of Lead acetate and Zinc chloride.

F.2(a)



F.2(b)



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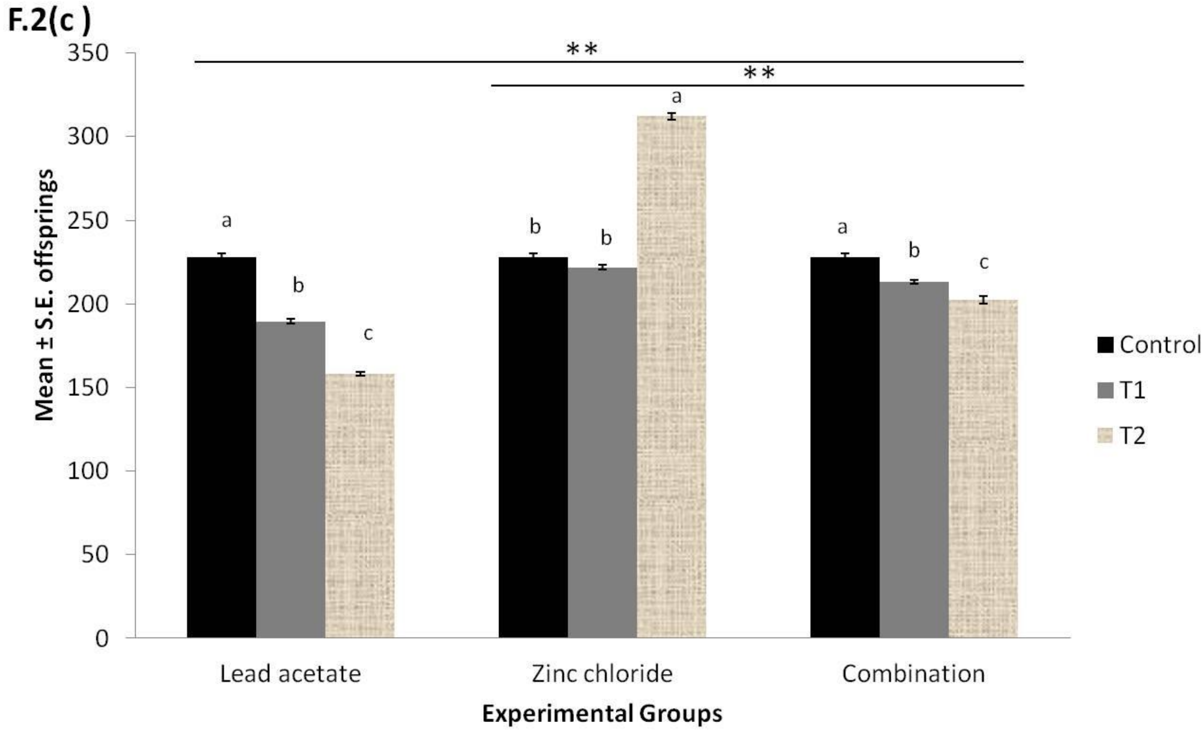
Correlation is significant at 0.01 levels (2- tailed)
Fig 2: Effect of Lead acetate and Zinc chloride on developmental time and viability of Drosophila melanogaster.



**** Correlation is significant at 0.01 levels (2- tailed)**
Alphabets a, b, c indicates significance of difference between control and treatment groups at p<0.05 level by Tukey's HSD test.
Fig 2(c): Fertility- measured as the average progeny produced for a period of 10days post mating

4. DISCUSSION AND CONCLUSION:

Environmental pollution due to heavy metal contamination has long been associated with deteriorating human health with Lead playing a key negative role. As the saying in native Kannada goes '*Athiyaadare amruthavu visha*' even useful factors of our day-to-day life can impose a threat if in excess. An example for this is the inappropriate diet followed by people resulting in health problems. Our study unveils the combined effect of heavy metals Lead and Zinc intoxication on fitness of *Drosophila melanogaster*. The *Drosophila* life cycle provides wonderful opportunities to unveil the possible trade-offs between its life-history traits with respect to metal toxicity. Though conflicting results have appeared on lead and zinc induced toxicity in the past, we believe that they reduce the fitness of organisms exposed. As hypothesized, our results show that the developmental exposure of excess Lead acetate and Zinc chloride together in *Drosophila melanogaster* hampers its fitness by delaying eclosion and reducing the viability of flies. But zinc seemed to show compensation for the reduction fertility caused by lead.

Responses to metals are dose dependant. Increase in the concentration of metals increases the mortality rates of the flies exposed. As suggested by Bonneton et al, (1996), one of the reasons can be because higher concentrations of metals can interfere with the necessary enzymes for hormone production. But determining the exact threshold is a difficult task as it can differ depending on the organism, environmental conditions, genetic constituency and chemical properties of the compound. As observed in the results, the concentration that caused death of 50% of the fly population (LC_{50}) varies between the compounds depending on their toxic impact. In our results, sub-lethal concentrations of lead acetate in the diet enhanced the developmental time and lowered the viability and fertility when compared to control. A deleterious effect on developing kidney in mice after exposure to lead acetate has been reported by Jabeen et al in 2010. Dhir and Dhand (2010) have already shown that chronic exposure to lead on female rats affected their reproductive performance with sustained poor fertility. Early reports by Sutron and Nelson (1937) showed that 0.5- 1% Zinc caused reduction in growth, anemia and poor reproduction in rats. Our results indicate inhibitory effects of zinc on the viability of fruitflies under laboratory conditions. In contrary to report by Sutron and Nelson we observed a stimulatory effect of zinc on the fertility of fruit flies. Developmental time did not seem to be affected by presence of zinc in media.

Developmental time is affected at-most by excess dietary lead and zinc in the form of lead acetate and zinc chloride when both present in the food media. It also reduced viability by >75% thereby showing a synergistic effect on the developmental viability of flies. Presence of zinc in the media alongside lead showed an increased fertility than lead alone in media. However this increase was only with respect to lead and not zinc as the fertility after combined exposure was still lesser than the single zinc exposure. Like us, previously Al Momami (2005) has shown that above 500ppm Lead and Zinc show significant decrease in survival, growth and development.

Thus to conclude, our study shows that excess dietary co-exposure of lead acetate and zinc chloride hampers the fitness in *Drosophila melanogaster* by prolonging the developmental time and reducing the viability of flies. Further a thorough molecular investigation might provide us the exact mechanisms underlying the interactions between lead and zinc and their role in causing intoxication.

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